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Genotyping dHT mice and real-time qPCR

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Updated date: Aug 29, 2022

An abbreviated version of this protocol was published in eLIFE in Mar 2022

Improvement of muscle strength in a mouse model for congenital myopathy treated with HDAC and DNA methyltransferase inhibitors

DOI: 10.7554/eLife.73718

Detailed protocol

Genotyping

- DNA Extraction (Phenol/Chloroform) --> ~ 4 hrs for 20 samples

Stock: **Lysis buffer 1x Lysis buffer 5x**
100 mM Tris pH 8 - 8.5 500 mM Tris pH 8
5 mM ETDA pH 8 25 mM EDTA pH 8
0.2 M NaCl 1 M NaCl
0.2% SDS 1% SDS
keep lysis buffer at rt, otherwise SDS will precipitate
Proteinase K
10 mg Proteinase K in 1 mL H2O (prepare fresh)
Hood: Alarm set button, Hi-Lo button
mouse line: 258 : Ex36ex91 (hetero)
Procedure

1. Get sample (**small**: mouse finger, **big**: piece of tail) (fridge lower drawer)
2. Mix lysis buffer 1x with proteinase K stock (10 mg/mL)
   495 µL lysis buffer 1x + 5 µL proteinase K stock (-> 0.1 mg/mL)
   small: 250 µL per sample big: 500 µL per sample

If amount of sample is very low, adapt TE volume at the end to increase DNA concentration

1. Add appropriate amount of mix to sample (make the samples float)
2. Incubate overnight (min. 4 h) @ 65°C / 1200 rpm in thermo shaker
3. Precool centrifuge in B2 to 4°C and (alu-rack to -20°C)
4. Lower temperature to 50°C for step 19 if continuing directly
5. Spin down samples
6. **Hood !** : Add phenol/chloroform (**lower phase**) (make personal aliquots) and mix 5 min on TOMY micro tube mixer (gel lab). Be sure that the tubes are mixing correctly or change for another hole. **NOTE**: before starting mixer put boxes with mice out of the room.
   small: 80 µL big: 100 µL
7. Vortex and centrifuge 5 min @ 4°C full speed. Be careful of the 2 phases.
8. *Transfer upper phase (small: **~240 µL** / big: **480 µL**) to new 1.5 mL tube. **200 µL** are enough*
   upper phase: aqueous phase with DNA lower phase: phenol (trash)

If DNA phase cloudy not a problem

1. **Add isopropanol** (small: **~260 µL** / big: **520 µL**) and mix well by hand **directly**. Don't touch the boarders with tips. **200 µL** are enough
2. Check DNA precipitate (break at this stage possible, store at -20°C)
3. Vortex and centrifuge 15 min @ 4°C full speed
4. Look for pellet and discard supernatant by pouring off (phenol waste in Falcon tube) / re-spin (1 min, 4°C, full speed) and remove last drop by pipette or using clean tissue. Cool in precooled rack (pellet more stiff)
5. Wash with **EtOH for RNA 70% (30% H2O for PCR)** (small: **~180 µL** / big: **360 µL**), quick vortex
6. Centrifuge 5 min @ 4°C full speed
7. Remove supernatant by pipette carefully
8. Dry pellet for 5 min @ 50°C on thermo shaker (open lid). 3-4 minutes are enough
9. Resuspend pellet in **60 µL TE buffer** (Tris, EDTA pH 8)
10. Dissolve well on thermo shaker @ 50°C for ca. 1 h 750 rpm
11. Vortex, quick spin, measure DNA concentration on NanoDrop (dsDNA)

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PCR

Master mix: samples (n + 1)
5x PCR Buffer (+Mg^{2+}) 5.0 µL
2.5 mM NTP 2.5 µL
Primer fwd (10 µM) 0.5 µL
Primer rev (10 µM) 0.5 µL
H_2O 14.0 µL
Taq Pol - GoTaq Promega 0.5 µL

DNA template (200 ng/µL) 2.0 µL
Total Volume 25.0 µL

PCR program:
Enzyme digestion (in case that is necesary)

Expected bands:

- WT allele: calculated: **450** bp observed: ~ **400** bp
- Het allele: calculated: **610** / **450** / **240** / **140** bp observed: ~ **575** / **370** / **255** / **165** bp

qPCR: Regular cDNA & qPCR (qPCRr)
All steps under hood
Vorrat: shelf below pH meter
Housekeeping gene: GAPDH
Preparation

1. Precool centrifuge to 4°C and the big one on 4th floor
2. A box of dry ice for samples
3. Put forceps on ice and clean with EtOH between each sample
4. Move Polytron to hood and pre-clean blade:

- **1X with EtOH and**
- **2X PCR H2O**

1. Reserve PCR (post-it) and if done on same day qPCR machine (online)

RNA isolation

1. Prepare n + 2 Falcon tubes (15 mL) containing ~ 7 mL PCR grade water (verser depuis la bouteille sans toucher le flacon, eviter falcon pipettes that are not RNA free).
2. Prepare 2 (+n depending on different genotypes) Falcon tubes (15 mL) containing 3 mL 70% EtOH (for PCR) in PCR grade water.
3. Prepare n Falcon tubes (15 mL) one per sample, label, tare and cool on dry ice.
4. Cut off a small piece of biopsy (~ 1 mm^3) (human samples) on Parafilm with new scalpel for each sample, weigh and push it to the bottom of the tube (work on dry ice, or very quickly to avoid degradation)
5. Wash blender (big blade in gel room) with 70% EtOH and PCR water in the beginning under hood.
6. Add 1 mL of TRIzol reagent (fridge near B2, keep an eye on stock) per Falcon and put on ice.
7. Transfer with cooled forceps the sample to the Falcon containing TRIzol.
8. Blend on ice for 1 min until all is homogeneous using big blade, do for all samples (keep on ice, homogenize completely) and put on ice.
   1000 µL Trizol: **muscle > 10 mg (EDL/Sol)** | 750 µL Trizol: **5 - 10 mg (EOM)** | 500 µL Trizol: **1 < 5 mg (Myotubes)**
9. Wash blender (big blade in gel room) with PCR water between each samples and trash the used Falcon with PCR water. Wash with 70% EtOH and PCR water between different genotypes and at the end. Get rid with excess of water by removing and shaking the blade and/or with a fresh paper tissue.
10. Clean blade in 5M NaOH (leave for 1 h, max. overnight)
11. Leave homogenate @ rt for 5 min
12. Transfer to 2 mL PCR epi tubes (RNase free)
13. Continue with procedure according to **"TRIzol reagent protocol"** for RNA

Homogenize sample:

1. Incubate at rt for 5 min (dissociation of nucleoprotein complex)
2. Add **chloroform** (1000 µL Trizol: **200 µL** | 750 µL Trizol: **150 µL** | 500 µL Trizol: **100 µL**) with Multipipette

Chloroform stock : pink room “RNA drawer”

1. **Shake** vigorously by hand for **15 sec** and incubate 2 - 3 min at rt + quick vortex.

2. **Centrifuge** at 12'000 x g (=rcf) for 15 min at 4°C, prepare Epi tubes -> separates into top colorless aqueous phase (RNA), interphase and lower red solvent phase (protein and DNA)
3. **Separate** top aqueous phase into new 1.5 mL tube**, avoid any inter- or lower phase. NOTE:** very important for good RNA purity, if necessary leave a bit of aqueous phase.

RNA precipitation:

1. *After quick spin down add 5 - 10 µg RNase-free Glycogen (drawer "qPCR" fridge hallway) (Thermo, R0551, 20 µg/µL, use **0.5 µL**) as carrier to aqueous phase (up and down)*
2. Add **2-propanol** (1000 µL Trizol: **500 µL** | 750 µL Trizol: **375 µL** | 500 µL Trizol: **250 µL**)
3. **Shake** vigorously by hand + vortex for **15 sec** and **Incubate** at room temperature for 10 min **(BREAKING POINT)**
4. **Centrifuge** at 12'000 x g for 15 min at 4°C (a pellet should be observable)

RNA wash:

1. **Remove supernatant** by pouring off to isolate RNA pellet
2. **Re-centrifuge** at 12'000 x g for 1 min at 4°C again to completely get rid of all supernatant (Phenol or Guanidine contaminations)
3. **Wash** with **75% EtOH** (1000 µL Trizol: **1000 µL** | 750 µL Trizol: **750 µL** | 500 µL Trizol: **500 µL**) no up and down here. **(BREAKING POINT)**
4. **Shake** vigorously by hand, quick vortex, and **Centrifuge** at 7500 x g for 5 min at 4°C
5. **Discard** the wash + vortex
6. *Optional: 2nd **wash with 75% EtOH** (1000 µL Trizol: **1000 µL** | 750 µL Trizol: **750 µL** | 500 µL Trizol: **500 µL**) no up and down here.*

***Shake** vigorously by hand, quick vortex, and **Centrifuge** at 7500 x g for 5 min at 4°C*
***Discard** the wash + vortex*

1. **Centrifuge** at 7500 x g for 3 min at 4°C.
2. **Remove as much remaining supernatant as possible with pipette.**
3. **Air-dry** (let tube open) pellet for 5 min (no longer!). Switch on hood!
4. **Resuspend (up and down)** the pellet in 30 µL (20 - 50 µL) pcr-grade water
5. Measure RNA concentration on **NanoDrop.**
6. *Optional: Remove DNA according to **"Protocol for DNase treatment"** if required (Life technologies 18068-015 in -20°C outside 408)*
   17 µL RNA + 2 µL DNase 10x buffer + 1 µL DNase
   Mix, spin down and incubate 15 min @ rt
   Add 1 µL of 25 mM EDTA
   Vortex, spin down and incubate 10 min @ 65°C
   Cool samples and continue or store at -20°C
7. Measure RNA concentration on NanoDrop (~ 300 ng/µL is normal) and write on epi
   A260/A280 ratio > 2.0 for pure RNA (> 1.8 is acceptable), > 1.8 for pure DNA
   A260/A230 ratio > 2.1 for pure RNA, > 1.7 for pure DNA
8. Store @ -80°C max 24 h (DNA is more stable than RNA)

Chloroform trash:

- Falcon chloroform trash: green trash
- Epis: put in minigrip and green trash

cDNA generation (Kit 4368814 suitable for **≤ 2 µg** RNA)
Work on ice until PCR amplification

1. Calculate amount required for 1000 ng RNA for each sample. If less is available take appropriate amount (500 ng or 200 ng, **remember correct dilution factor!!**)
2. Calculate volume of PCR water to complete for 14.2 µL
3. Prepare 0.2 mL PCR epi tubes (RNase free) per sample
   Write sample name and "reg." for regular cDNA on lid & side
4. Prepare master mix according to protocol **"High Capacity cDNA Reverse Transcription Kits Protocol"** for n + 1 sample (kit 4368814 Applied Biosciences in -20°C outside 408 2nd drawer), **cool on ice!**
   2.0 µL RT buffer 10x
   0.8 µL dNTP mix 25x (100 mM)
   2.0 µL RT random primers
   1.0 µL Multiscribe rev. transcriptase
   5.8 µL per sample
5. Add calculated amount of water, then **5.8 µL** master mix and finally calculated amount of sample, mix by pipetting up and down (1000 ng RNA, in 20 µL final volume)
6. Run "cDNA" program on PCR machine # 2 (duration 2,5 h, stored under user "cDNA"):
   10 min @ 25 °C
   120 min @ 37 °C (set to 3 x 20 min @ 2 cycles)
   5 min @ 85 °C (-> inactivate enzyme)
   ∞ @ 4°C

enter **20 µL** reaction volume

1. Close lid and start
2. After completion store in mini-grip @ -20°C

qPCR (10 ng cDNA used, Primer conc: 250 nM)
make sure qPCR device is free : 1 plate --> ABI 2 2 plates --> ABI 5-6
3-4 controls and always duplicates! WT and HT always on 1 plate!

1. *Prepare fwd & rev primers (10 µM dilution, if not present, make dilution in PCR water from 100 µM -> **10 µL** & **90 µL** PCR grade water) stored in -20°C in 408*
   Use muscle specific primers to normalize (actinin, desmin)
2. Prepare target mixes for n + 2 samples (analyzed in duplicates) containing the following per sample (don't vortex but mix by hand and centrifuge):
   10 µL Power Up Sybr Green PCR master mix (A25742 in -20°C outside 408)
   9 µL PCR water (rel. fresh)
   0.5 µL fwd primer (250 nM)
   0.5 µL rev primer (250 nM)
3. Arrange plate setup in Excel and print sheet (primer duplicates in rows, samples in columns):

1. Prepare 96-well plate (life techn. 4346906) on black background (sheet of dark paper)
2. Add **20 µL** of **target mix** in proper wells (ACTN2 in rows A & B, DES in rows C & D, ...) using automated pipette with PCR 500 µL Combitips inserting it to bottom of 96 well plate
3. Add **2 µL** of **diluted sample (≙** 10 ng cDNA):

Be careful to not heat the samples with fingers.
Use PCR water instead of sample for negative control

1. Seal with foil without any wrinkles
2. **Quick spin down (1000 rpm) on centrifuge (in MilliQ water system room, prog. 5 on centrifuge, reclick on it after spinning) → measure**

How to cite:(Readers should cite both the Bio-protocol preprint and the original research article where this protocol was used)

1. Zorzato, F. and Ruiz, A. (2022). Genotyping dHT mice and real-time qPCR. Bio-protocol Preprint. bio-protocol.org/prep1881.
2. Ruiz, A., Benucci, S., Duthaler, U., Bachmann, C., Franchini, M., Noreen, F., Pietrangelo, L., Protasi, F., Treves, S. and Zorzato, F.(2022). Improvement of muscle strength in a mouse model for congenital myopathy treated with HDAC and DNA methyltransferase inhibitors. eLIFE. DOI: 10.7554/eLife.73718